AD_____

Award Number: W81XWH-07-1-0065

TITLE:
 Effects of radiation on proteasome function in prostate cancer cells

PRINCIPAL INVESTIGATOR:
Frank Pajonk, M.D., Ph.D

CONTRACTING ORGANIZATION:

 University of California,
Los Angeles, CA 90024

REPORT DATE:

February 2010

TYPE OF REPORT:

Annual Summary Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

      ☑ Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
01-02-2010	Annual Summary Report	15 JAN 2009 - 14 JAN 2010

4. TITLE AND SUBTITLE

Effects of radiation on proteasome function in prostate cancer cells

5a. CONTRACT NUMBER

5b. GRANT NUMBER

W81XWH-07-1-0065

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Pajonk, Frank

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University

Of California, Los Angeles,

CA 90024

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research
And Material Command
Fort Detrick, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

No abstract provided.

15. SUBJECT TERMS

No subject terms provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	UU	13	**19b. TELEPHONE NUMBER** (include area code)

Table of Contents

Introduction

The proteasome is involved in the progression of the cell cycle and its degrading activity controls the lifetime of most cellular proteins, including many regulatory proteins. This multicatalytic complex is also the main target of many cancer therapies, including radiation, and our lab has already extensively shown data on radiation-induced proteasome inhibition. Based on the observation that radiation causes chemical modifications to many proteins and/or enzymes present in a cell through free radicals production, we hypothesized that irradiation induces structural, other than functional changes within the 26S subunits and proteins interacting with it. These conformational changes are the ones that eventually affect its activity, thus its capacity of degrading proteins.

As a cell progresses through the cell cycle-specific proteins need to be degraded in order for the cell to proceed to the next phase. So far, the activity of the 26S proteasome was thought to be constant throughout the different phases of the cell cycle, and that the degradation of specific proteins was only dependent on their ubiquitination and not by the activity levels of the proteasome itself. Our hypothesis is that there is another level of regulation that happens as the cell progresses through the cell cycle. This level is mediated by regulating the degradation efficiency of the proteasome itself. If this were in fact true, than cells would dynamically change the state of the protesome through the cell cycle, which could explain why radiation can only partially inhibit proteasome function if the susceptibility of the proteasome to radiation changes with its regulation.

Body

As already described in our last progress report we were able to establish a protocol for immunoprecipitation of intact 26S proteasomes from prostate cancer cells (PC-3 cells). Using the expression vector given to us by Dr. Lan Huang from UC Irvine, we were able to tag one of the 19S proteasome subunit (Rpn11) and use it as a bait for the pulling down the intact 26S proteasome complex.
After having reported on all the proteins associated to the proteasome identified by mass spectrometry in irradiated and un-irradiated samples, we decided to focus on the principal components of the 26S proteasome. This complex is composed of at least 28 subunits that reach a molecular weight of almost 2.5MDa. Initially, our protocol for the precipitation of the 26S proteasome involved the use of streptavidin magnetic beads binding the tagged proteins. In later experiments we substituted the magnetic beads with high capacity agarose beads (Upstate beads), to increase sensitivity and efficiency and to reduce the time required for the pull down.

The resolution of the SDS-PAGE gel previously performed in our previous studies was not high enough because the molecular weights of almost all subunits are very close to each other. The same problem was faced using a chromatographic approach and running pure proteasome preparations on an LC-MS system (see previous progress report). In order to overcome this difficulty, we performed two-dimensional electrophoresis (2D-E) on 26S proteasome preparations. Briefly, cells were plated and grown to confluence. Cells were subjected to irradiation (10 Gy) at room temperature using an X-Ray source or sham irradiated. Cells from the two groups (irradiated and non-irradiated) were lysed and proteins quantified. At the same time, streptavidin agarose beads were washed and activated accordingly with the manufacturer's instruction and incubated with the sample overnight at 4 ˚C. After incubation, samples bound to the beads were washed twice in lysis buffer completed of 1x phosphatase inhibitor (Sigma), 1x protease inhibitor (Sigma) and TEB buffer (Tris-HCl, pH7.5). Samples were dissolved in urea/(8M) thiourea (2M) buffer (high denaturing buffer) for the dissociation of the 26S proteasome from the beads. After centrifugation, samples were collected, concentrated and precipitated in 100% cold acetone. Proteins were finally dissolved in rehydration buffer (urea/thiourea buffer). The amount of proteins (26S proteasomes) was quantified using 2D-Quant kit (Amersham). Briefly, precipitated proteins are

resuspended in a copper-containing solution and unbound copper is measured with a colorimetric agent. The color density is inversely related to the protein concentration. The standard curve was generated by using bovine serum albumin as a standard protein. The assay has a linear response to protein in the range of 0-50micrograms, allowing the measurement of even a very low quantity of proteins.

25 micrograms of 26S proteasomes from irradiated and un-irradiated samples were loaded on a 11cm agarose strip (BioRad) for their separation based on their isoelectric point (pI, first dimension). Strips were hydrated and incubated in equilibration buffer added with DTT and iodoacetamide for the reduction and alkylation of disolfure bounds. Precast polyacrylammide gels (Criterion Precast gel) were used for the second dimension. Gels were stained for phosphorylation signals using the ProQDiamond dye (Amersham) and, successively, with Sypro Ruby (Amersham) for the identification of the total proteins. Both staining were performed following manufacturer instructions (Amersham). A Typhoon 9400 scanner (Amersham) was used to scan the gel after phosphoryaltion (ProQ diamond dye) and total protein staining (Sypro Ruby). Fig.1 shows 2D gels from 26S proteasomes of PC3 un-irradiated (left gel) and irradiated (10Gy) (right) stained with ProQ Diamond for the detection of differences in phosphorylation.

pH3   pH10 pH3   pH10
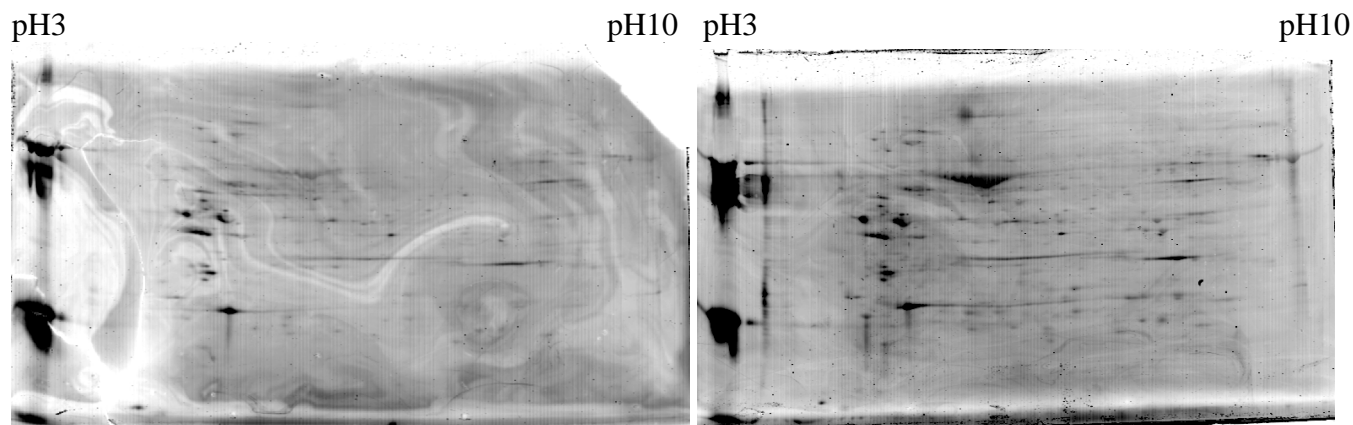

Fig 1. 2D gels from 26S proteasomes of PC3 un-irradiated (left gel) and irradiated (10Gy) (right) stained with ProQ Diamond for the detection of differences in phosphorylation

In order to characterize proteins differentially phosphorylated in irradiated and un-irradiated samples we already picked spots, digested them with trypsin using the protocol already established. We currently run them on a mass spectrometer instrument for their identification.

One technical issue in this experiment is the weak sensitivity and reproducibility of the phospho-staining probably due to the intrinsic characteristics of dye. To overcome this issue, we decided to isolate the 26S proteasomes as previously and to identify phosphorylation sites by using specific antibodies. In particular, we are using 3 different antibodies directed against domains containing amino acids subjected to phosphorylation (anti phospho-tyrosine, phospho-threonine and phospho-serine; all antibodies are from Abcam). Briefly, proteasomes from irradiated (10Gy) and non-irradiated cells are pulled down from the total lysate using streptavidin beads, run on a first dimension (for the separation based on the pI), and on a 2D gel (for their separation based on the molecular weight) as in the protocol described above. 26S proteasomes are next transferred from the 2D-gels to PDVF membranes at 100V for 1h on ice. Membranes are blocked by 3% BSA in PBS-Tween for 1 hour at room temperature and stained with antibodies for phosphorylation sites (1h at room temperature for both primary and secondary antibodies). So far, we have confirmed an increase of phosphorylation in the irradiated samples compared with the non-irradiated samples. However, this study is currently in progress and data are still very preliminary.

As mention in the introduction section, the proteasome plays a pivotal role in the progression of the cell cycle and its activity is currently thought to be static throughout the different phases of the cell cycle. We hypothesized that this is not the case and that regulation of the proteasome during the cell cycle alters its susceptibility to radiation.

In order to test our hypothesis, we synchronized PC-3 cells. There are several different techniques reported in the literature for synchronizing cells in various stages of the cell cycle. Balancing between a synchronized cell population and the toxicity exerted from some commercially available compounds, we decided to double block the cell cycle by treating cells with thymidine and mimosine. Mimosine is a L-amino acid found in large quantities in the foliage and the seeds of *Laucena glauca* and *Mimosa pudica*. It is a reversible inhibitor of DNA synthesis with a general anti-mitotic activity that affects both the initiation and the elongation phases of the DNA synthesis. Thymidine is a cell cycle inhibitor acting through a feedback inhibition of the nucleotide synthesis. Briefly, cells were plated in 10cm dishes, grown for 36 hours and subsequently treated for 12hours with an excess of thymidine (2mM in regular media). This concentration blocks the cells in G1/S boundary. Cells were released and let cycle for 9 hours, time necessary to exit the G1 phase, go to S phase and enter in G2/M phase. Next, cells were treated with Mimosine at 400 µM for 12hours. For each time point a control dish (cells normally cycling plated at the same time and density as the synchronized dishes) was added. All the values data from the different time points were normalized to the corresponding controls. Time points of 0h, 3h, 6h, 12h and 24h were chosen to cover an entire cell cycle.

Proteasome function assays for the three leading activities were performed at each time point for the treated cells and their corresponding controls. The change of the leading chymotryptic 26S proteasome activity is shown in fig. 2.
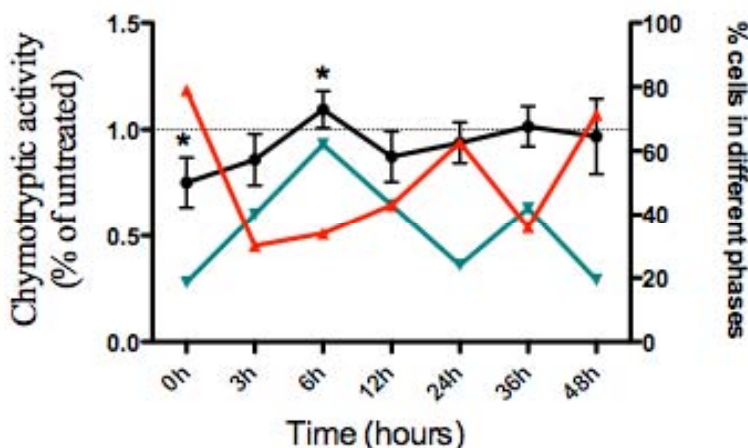

*Figure 2: Proteasome function assay and cell cycle analysis in synchronized PC-3 cell line expressing the hRpn11 HTBH tag. Data were normalized to control cellsfor each time point. The black line represents the chymotryptic activity. Means and SEMs are shown from at least three different experiments. *: p<0.05. The 26S proteasome activity significantly dropped in synchronized cells (0h) and increased with the progression of the cell cycle (12h). The red line indicates the percentage of cells in G1 phase of a representative experiment. The green line indicates cells in G2/M phase.*

DNA analysis by FACS was also performed for each time point for both, controls and treated cells. For this purpose, cells were harvested, fixed in 70% ethanol and frozen at -20C for at least one night. An example of cell cycle analysis from synchronized PC-3 cells is shown in fig. 3.
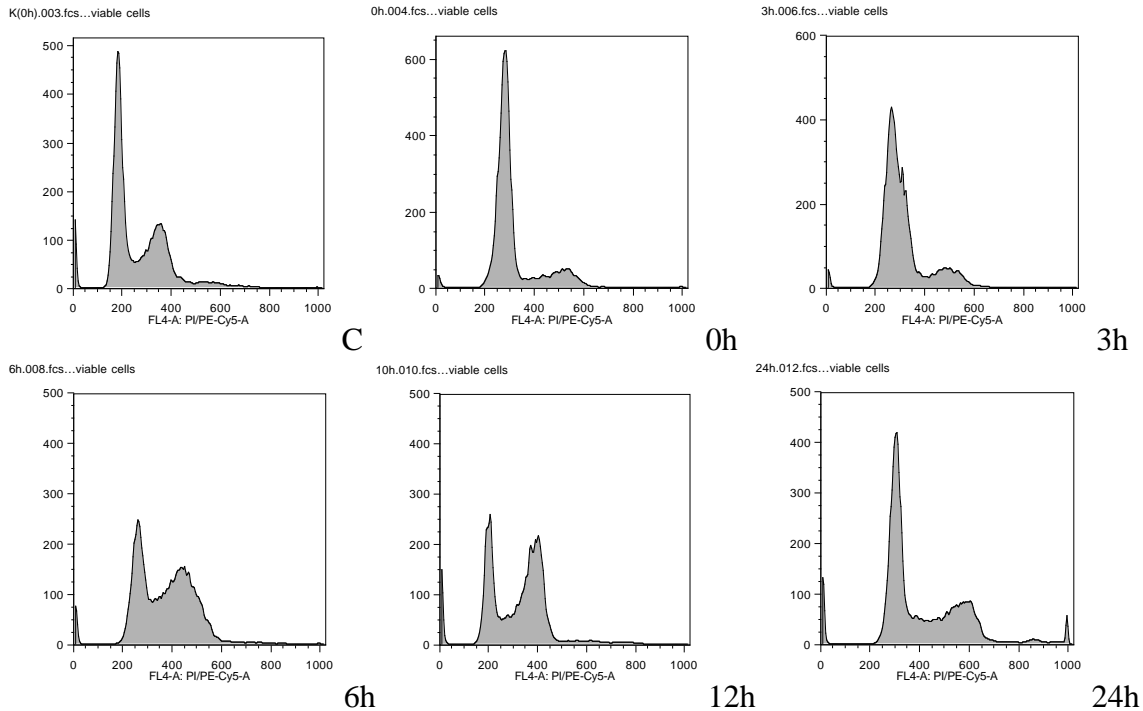

Fig 3. Representative cell cycle analysis of synchronized PC-3 cells by FACS. The hours indicate the amount of time elapsed since restarting of the cell cycle. "C" stays for untreated control cells.

As indicated in the panels above the adopted protocol allowed us to synchronize PC-3 cells. At 0h and 3 hours the majority of cells was in the G1 phase. At 6 hours, an increase in the number of cells in S phase was observed and reached its maximum at 10h. At 24hours, cells had redistributed.

Our preliminary results show a decrease of chymotryptic activity as the cells progress through the cell cycle. In particular, cells at the G1/S phase transition show a drop of activity at 0 ad 3 hours. In order to investigate mechanisms of this drop of chymotryptic activity, we pulled down 26S proteasomes at the time point at which we registered the maximum decrease of activity as described above (agarose streptavidin beads). 26S proteasomes from cells normally cycling and cells still synchronized in boundary G1/S phase were analyzed by two dimensional gel electrophoresis (see protocol above) using 11cm strips (BioRad) for the first dimension and pre-casted gradient gels (Criterion-BioRad) for the second dimension (see protocol described above). Gels were stained with ProQDiamond for the detection of differences in the phosphorylation status and, successively, with Sypro Ruby stain for the detection of the total amount of proteins. Results from this analysis are shown in fig. 4.
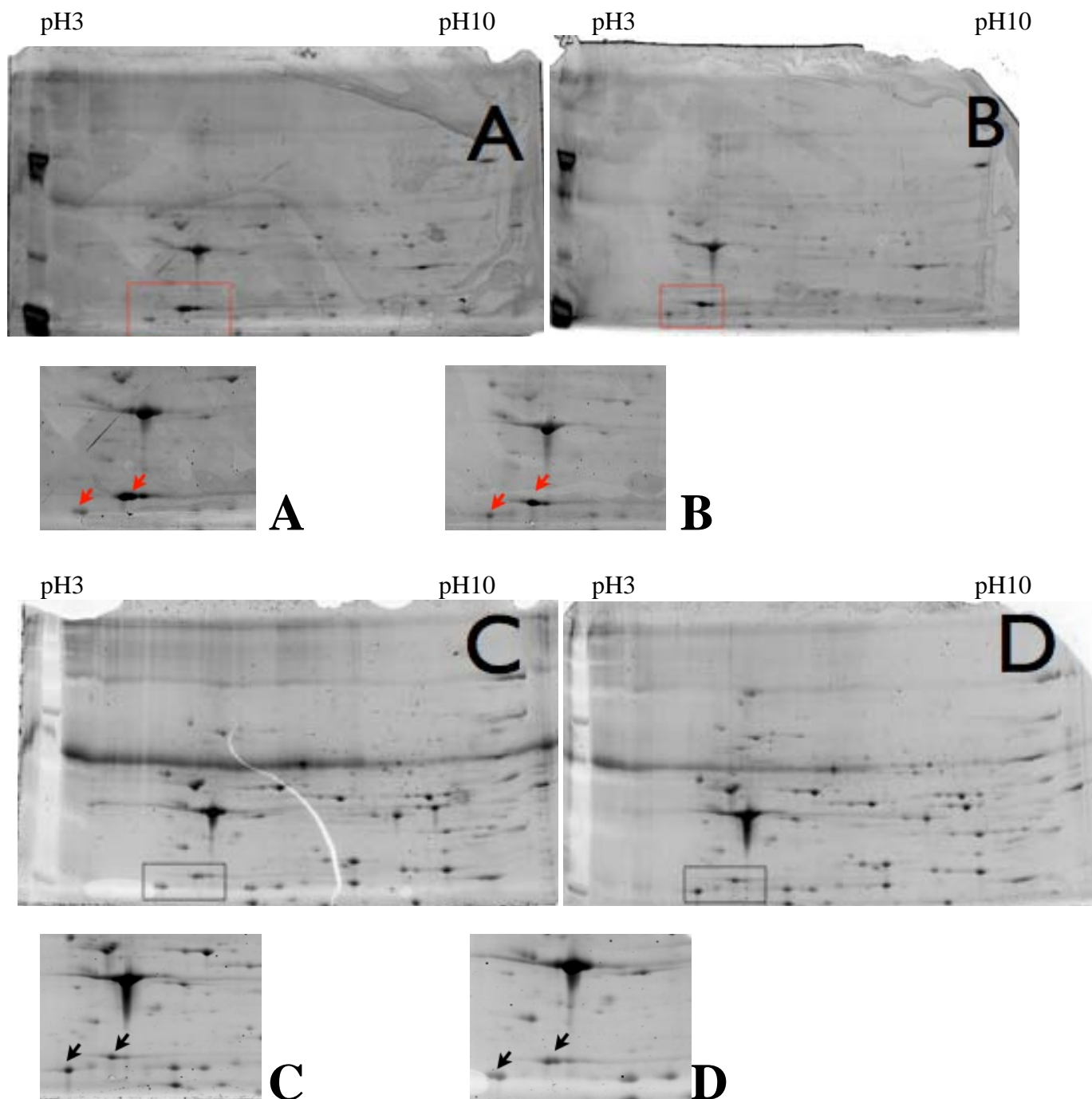
Figure 4. Two-dimensional gel electrophoresis (2DE) of 26S proteasome preparations from PC3 cells expressing hRpn11 HTBH tag. Samples were taken from cycling cells (A and C) and synchronized cells (B and D). 26S proteasome preparations were obtained as indicated in Material and Method section; 20 microgram of pure 26S proteasome preparations were used. The gels were stained with proQ Diamond and with Sypro Ruby stain. On the top left, figure A, 26S proteasome subunits obtained from cycling cells stained with ProQ Diamond for the identification of phosphorylated subunits. On the top right, figure B,

26S proteasome subunits obtained from synchronized cells and stained with ProQ Diamond for the identification of phosphorylated subunits. On the bottom left, figure C, 26S proteasome subunits from cycling cells stained with Sypro Ruby. On the right, figure D, 26S proteasome subunits from synchronized cells stained with Sypro Ruby.

Spots (26S *proteasome* subunits) from synchronized cells in G1/S phase transitions and normally cycling cells were found to be different in respect of their phosphorylation status as indicated by the black arrows in figures C (control) and D (synchronized cells). These spots were cut, digested with trypsin and analyzed by mass spec with an FT/MSMS instrument for their identification and for the detection of the phosphorylation sites. Mass spectrometry analysis was carried out at the Pasarow Mass Spectrometry Lab, UCLA.

Differences were identified for two proteasome subunits found in the 20S proteasome core particle: alpha 3 and alpha 5. The spectrometral analysis showed the presence of phosphorylated amino acids only in one (alpha 3 subunit) of the two spots analyzed. Mascot analysis matched the alpha 3 subunit with 48% sequence coverage and a score of 893 (PSMA3; accession number: P25788). Phosphorylation of tyrosine (seventh amino acid of the sequence and first tyrosine residue: M.SSIGTG<u>Y</u>DLSASTFSPDGR.V) of this subunit was found in cells normally cycling. Tyrosine phosphorylation was lost when cells were arrested at the G1/S phase boundary and instead the subunit was phosphorylated at serine 2 (second amino acid of the sequence and second serine residue: M.S<u>S</u>IGTGYDLSASTFSPDGR.V). We are currently exploring the mechanism behind this post-translational modification.

An alternative explanation for our inability to reduce proteasome function by radiation by more than 50% is a possible heterogeneity of the cells regarding their proteasomes. To study this possibility, we engineered PC-3-Rpn11 cells to express a fusion protein of ZsGreen and the c-terminal degron of ornithine decarboxylase (cODC). The latter is recognized by the 26S proteasome, which leads to immediate, ubiquitin-independent degradation of the fusion protein through the proteasome. In contrary, cells with low proteasome activity show high ZsGreen expression and are detectable by fluorescence microscopy or flow cytometry. Fig 5 shows PC-3rpn11 cells infected with ZsGreen ODC vector.
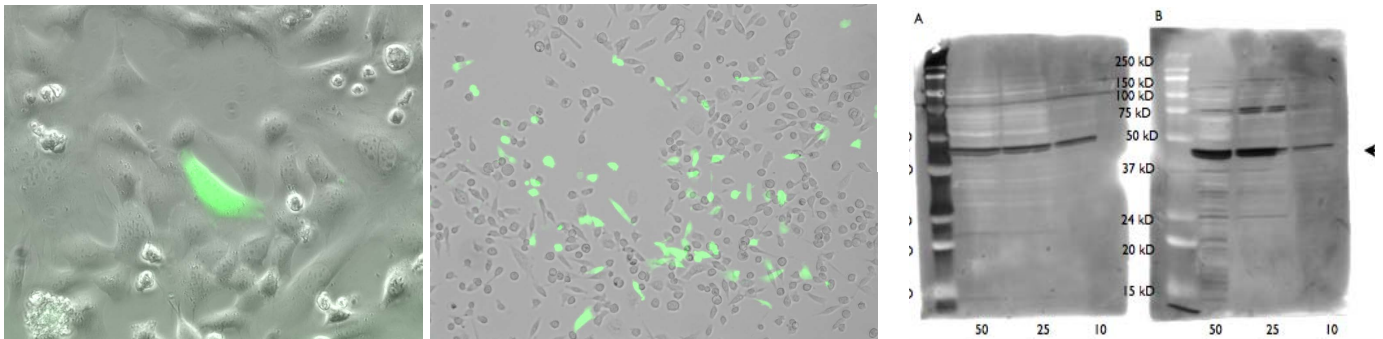

Figure 5. On the top representative microscopic image of PC3rpn11 expressing ZsGreen ODC. Bottom part: Western blot on Rpn11 (A) and 6xHis tag (B) in PC-3zsGreenODC cell line transfected with the retrovirus for the expression of hRpn11 HTBH tag. From the left: molecular weight marker, 50 microgram, 25microgram and 10micrograms of protein.

FACS analysis (Fig. 6) shows a percentage of cells cultured in monolayer positive for ZsGreen ODC equal to 9%.
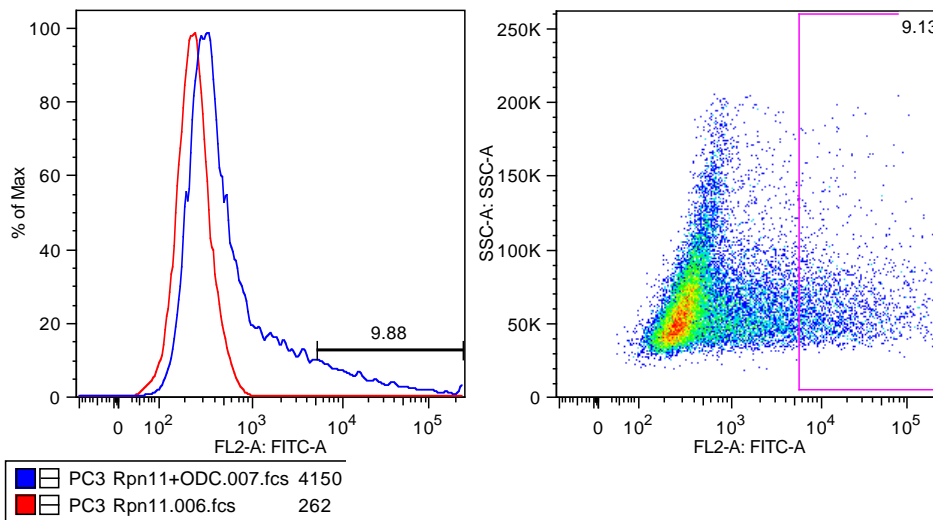
Figure 7. FACS analysis of PC-3 rpn11 and PC3-rpn11ZsGreen ODC. Cells transfected with ZsGreen ODC are highlighted in blue. Near 10% of cells are positive for ZsGreenODC (labeled FITC positive).

To study the structure of the 26S proteasome in the subpopulation of PC3 cells expressing high levels of ZsGreen, cells were sorted based on their ZsGreenODC expression in ZsGreenODC-negative and ZsGreenODC-positive cells.

Fig 7 illustrates the parameters for the sorting. Only highly positive ZsGreenODC cells (3% of the entire population) were considered for the pull down (labeled GFP+ in the figure).
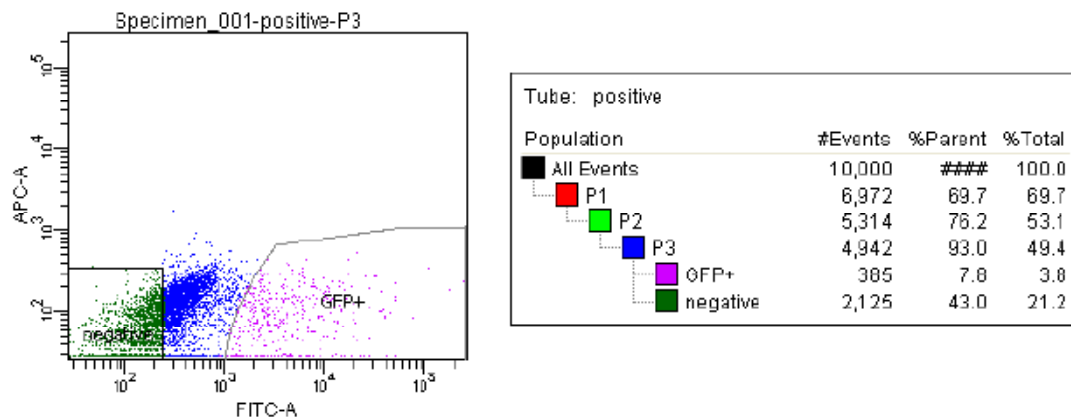

Figure 7. FACS analysis of PC-3 rpn11 and PC3-rpn11ZsGreen ODC (GFP+) for sorting (FACS facility, UCLA).

Proteasomes from both cell populations, PC3-ZsGreenODC-negative and -positive sorted cells, were pulled down with the technique described above, concentrated and precipitated in 100% acetone. The amount of proteins (26S proteasomes) was quantified. 25micrograms of pure 26S preparations from PC-3 ZsGreenODC-negative or PC3 ZsGreenODC-positive sorted cells were focused on 11cm strips for the separation based on their pH (IEF) and next loaded on a 2DE gradient precast gel (Criterion, BioRad) (second dimension, 2DE). Gels ran at 180Volts for 1 hour and half; a 2DE marker (BioRad) for the detection of the molecular weight was used. Gels were fixed overnight in 70% methanol, 10% acetic acid in

pure water (fixation buffer). Total protein was detected by staining with the Sypro Ruby dye for 6 hours in the dark, washed for 1 hour in destaining solution (methanol/acetic acid in water), and scanned

Fig. 8 shows our preliminary results. On the top, 26S proteasome preparation from PC3-ZsGreenODC-negative cells. On the bottom, 26S proteasomes from PC3-ZsGreenODC-positive cells. Gels were analyzed using the ImageJ software. Differentially expressed spots are highlighted in red and results from a semi-quantitative analysis are shown in table 1.
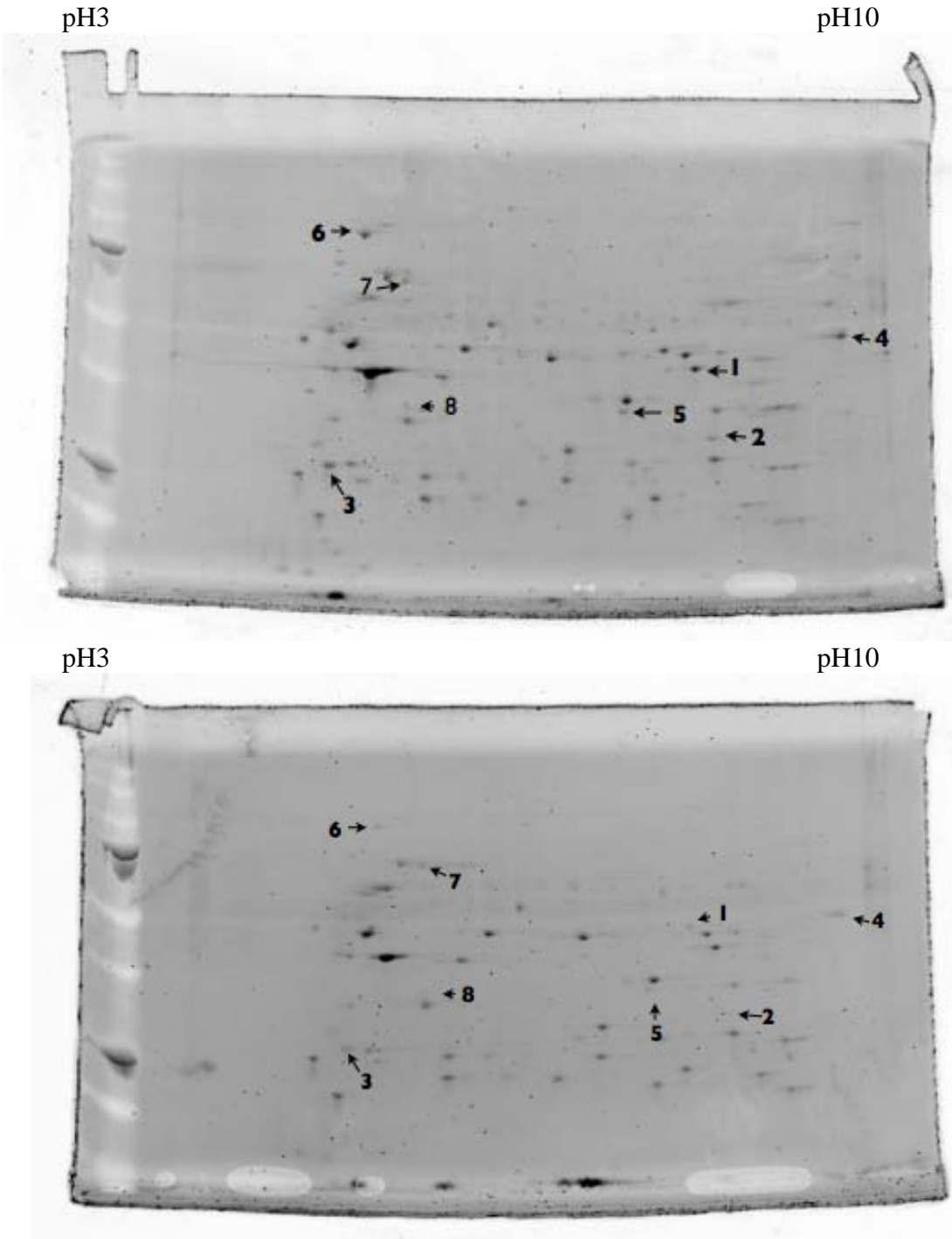
Figure 8. Two-dimensional gel of 26S pure preparations pulled down from sorted PC3 ZsGreenODC negative (top) and positive (bottom) cells. Gels were analyzed using ImageJ. Spots differently expressed between the two populations are highlighted with arrows in the figures. Table 1 shows the pixel intensity of each spot.

Table 1. Expression profile of the differentially expressed 26S subunits. Analysis was performed using ImageJ. Spot ID refers to spots in figure 8.

	PC3ZsGreenODC Negative	PC3ZsGreenODC Positive
Spot ID	Pixel intensity	Pixel intensity
1	152.3	109.8
2	126.8	98.3
3	129.6	121.1
4	141.7	104.1
5	119.1	104.1
6	134.2	99.9
7	125.2	115.1
8	105	NA

As shown in the upper table, our analysis revealed that proteasome subunits are in general expressed at lower levels in PC3-ZsGreenODC-positive cells (red bar in the graphs) compared to PC3-ZsGreenODC-negative cells.

These spots differentially expressed between the two samples have been cut from the gel, digested and are currently analyzed by mass spectrometer. Results will be confirmed by Western blot using antibodies already tested (Biomol).

Key Research Accomplishment:

1. Study of the 26S proteasome subunits profile with proteomics tools using the biotin-tagged Rpn11 subunit.

2. Discovery that 26S proteasome activity is tightly regulated during the cell cycle, being down-regulated at the G1/S phase boundary.

3. Identification of 26S proteasome subunits being post-translational modified in different phase of the cell cycle.

4. Establishment of a prostate cancer cell line (PC3) transfected with a tagged Rpn11 subunit and a ZsGreen-cODC construct to investigate proteasome heterogeneity in prostate cancer cells.

5. Establishment of a protocol for studying the phosphorylation status of by blotting two-dimensional gel electrophoresis using specific phospho-antibodies.

Conclusion:

Our results so far have shown that the 26S proteasome is regulated thorough the cell cycle, and presents a drop in its activity in G1/S phase. The inhibition of its activity is probably due by post-translational modifications, and in particular phosphorylation of some of its subunits. Our proteomic tools (mass spectrometry and 2DE) have been optimized this year and represent an excellent tool for further

investigations. Our data showing a map of 26S proteasomes from a rare subpopulation of prostate cancer cells open a new important opportunity for understanding why many therapies still fail in curing cancer and how their efficacy can be increased by targeting the right cell subpopulation.

Reportable outcomes:

Vlashi E, Kim K, Dealla Donna L, Lagadec C, McDonald T, Eghbali M, Sayre J, Stefani E, McBride W, Pajonk F: *In-vivo imaging, tracking, and targeting of cancer stem cells.* J Natl Cancer Inst 2009, 101:350-359.

Pajonk F, Vlashi E, McBride WH: *Radiation Resistance of Cancer Stem Cells - The 4R's of Radiobiology Revisited. Stem Cells* 2010. ePub ahead of print

Vlashi E, McBride WH, Pajonk F: *Radiation responses of cancer stem cells.* J Cell Biochem 2009, 108:339-342.

Poster presentation at the AACR 10th Annual Meeting (Denver, CO) Lorenza Della Donna, Erina Vlashi, Chann Lagadec, Julian Whitelegge, Puneet Souda, Polin Nikolay, Malcom Mattes, William McBride and Frank Pajonk. *Quantitative proteomic analysis of radiation induced posttranslational modifications of the 26S proteasome, 2009*